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Biodiversity of Living, Non-marine, Thrombolites of Lake Clifton, Western Australia

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Abstract

Lake Clifton in Western Australia is recognised as a critically endangered ecosystem and the only thrombolite reef in the southern hemisphere. There have been concerns that increases in salinity and nutrient run-off have significantly impacted upon the thrombolite microbial community. Here we used cultivation independent molecular approaches to characterize the microbial diversity of the thrombolites at Lake Clifton. The most dominant phyla currently represented are the *Proteobacteria* with significant populations of *Bacteroidetes* and *Firmicutes*. *Cyanobacteria*, previously invoked as the main drivers of thrombolite growth, represent only a small fraction (~1-3% relative abundance) of the microbial community. We report an increase in salinity and nitrogen levels at Lake Clifton that may be contributing to a change in dominant microbial populations. This heightens concerns about the long-term health of the Lake Clifton thrombolites; future work is needed to determine if phyla now dominating this system are capable of the required mineral precipitation for continued thrombolite growth.

Keywords: microbialite, thrombolites, diversity, sequencing

Introduction

Microbialites are biosedimentary structures formed by the interaction of microbial communities with their environment, and are found throughout large portions of the geological record. Stromatolites (layered) and thrombolites (clotted) are morphological types of microbialites, differentiated by their internal mesostructure, and have been cited as providing some of the earliest evidence for life on Earth almost 3.5 billion-years-ago (Allwood et al. 2006; Walter et al. 1980). These ancient ecosystems may have signalled the first appearance of cellular organization and photosynthesis (Carr and Whitton, 1973) and thus, they can potentially provide insights into the nature of habitable environments on the early Earth, the antiquity of microbial metabolisms and the evolution of biogeochemical cycles. Living microbialites are rather rare, found in just a few select locations worldwide, including the open marine waters of Exuma Sound, The Bahamas (Dill et al. 1986, Baumgartner et al. 2009), the hypersaline region of Hamelin Pool, Western Australia (Playford 1990, Reid et al. 2003, Goh et al. 2009) and the brackish waters of Lake Clifton, Western Australia (Wacey et al. 2010) that are the subject of this study.

Radiocarbon dating indicates that the age of the thrombolites in Lake Clifton is approximately 1950 years BP to modern (Moore and Burne 1994). They were probably formed after the isolation of the lake from the sea, which occurred between 4,670 and 3,890 years BP (Coshell and Rosen 1994), by precipitation of aragonite by microbial activity (Moore 1993). Lake Clifton has many unusual features including the existence of aquatic fauna (predominantly Crustacea, nematodes and some Protista (Konishi et al. 2001)) and no natural drainage channels - the lake is either replenished by winter rains or from underground water, with an extensive aquifer emptying into the lake along the eastern shoreline (Moore et al. 1984). This inflow of fresh groundwater provides calcium-enriched water critical to the survival of the micro-organisms and the growth of the thrombolites.

The thrombolite reef at Lake Clifton supports the largest living, non-marine microbialite community in the Southern hemisphere (Moore and Burne 1994). It has been suggested that the thrombolites have been produced by a combination of sediment trapping, sediment binding and precipitation resulting from the metabolic activity of communities of photosynthetic prokaryotes (including cyanobacteria), eukaryotic microalgae (e.g. diatoms) and chemoautotrophic and chemoheterotrophic microorganisms (Moore 1991). Historically the *Cyanobacteria* have been the most studied phylum in microbialite ecosystems, with the community of Lake Clifton thrombolite forming organisms being previously described as including filamentous (*Scytonema, Oscillatoria, Anabaena*) and unicellular (*Aphanocapsa, Chroococcus, Aphanothece*) cyanobacteria (Moore 1991). Moore and Burne (1994) later noted that the most abundant Cyanobacterium was *Scytonema* - a well-known, non-obligate calicifer requiring low nutrient conditions and fresh to brackish waters. Stable isotope analyses of the thrombolites suggested that the main process responsible for their formation was the photosynthetically influenced precipitation of aragonite predominately by *Scytonema* but also by other members of the benthic microbial community (Moore and Burne 1994). Knott et al. (2003) have raised concerns about the changing conditions in Lake Clifton where a dramatic increase in the salinity of the lake, dating back to at least 1992, has been observed. Given that thrombolite microbial populations (assessed by microscopy techniques available at the time) were thought to be dominated by *Scytonema,* which require low salinity and nutrient conditions for survival, it is suggested that the benthic microbial community will have been significantly affected by increases in salinity and nutrient concentrations. The thrombolites at Lake Clifton were placed on the critically endangered list in 2000 (Luu et al. 2004); given that the limnological processes of the lake are threatened by current human intervention, it has become critical that we increase efforts to elucidate the microbial diversity and consequent functioning of this unique ecosystem.

In more recent times considerable progress has been made using molecular-based approaches to characterise microbial diversity across a variety of microbialite ecosystems. These approaches have identified a taxonomically diverse consortium of organisms present in stromatolites (e.g. Havemann and Foster 2008; Goh et al. 2009), thrombolites (e.g. Myshrall et al. 2010; Mobberly et al. 2012) and microbial mats (Centeno et al. 2012). Characterisation of thrombolites present in The Bahamas suggests that they are dominated by bacteria (Myshrall et al. 2010), in particular the *Proteobacteria*, with the *Alphaproteobacteria* being the most dominant class (Mobberley et al. 2012). The authors also identified purple non-sulfur phototrophs (from the Orders *Rhizobiales* and *Rhodobacterale*), *Deltaproteobacteria* including the *Myxococcales* and the metabolically diverse sulfate-reducing bacteria of the order *Desulfobactererales* as being important within this ecosystem. Although cyanobacteria were present in abundance the system (Mobberley et al. 2012) it has been recognised that they are not the most abundant phylum across all microbialite systems (e.g. Centeno et al 2012.). The aim of the current study was to examine the microbial diversity of Lake Clifton thrombolites with particular reference to changes in water salinity and nutrient status that has taken place in recent years. We utilized cultivation independent molecular approaches, specifically next generation barcoded sequencing via the Ion Torrent Personal Genome Machine (PGM), to characterize the microbial diversity of the thrombolites at Lake Clifton in order to identify the dominant organisms within this habitat.

Materials and Methods

Sample Description and Sample Sites

Lake Clifton is situated around 100 km south of Perth in Western Australia (Figure 1a). It is 21.5 km long and up to 1 km wide, lying around 1-4 km inland from the Indian Ocean (Moore 1987). Most of the lake is less than 1.5 m deep, with some areas up to 3.5 m in depth.

Both the deep basin and the mean annual water level are generally lower than sea level (Moore, 1991). A reef of thrombolytic microbialites persists for around 6 km along the north-eastern shoreline extending up to 120 m into the lake (Burne and Moore, 1993). The thrombolite macro-morphology varies shoreward, from discrete domal and conical structures up to a metre in height, to tabular and coalesced structures with reduced topography (Figure 1b). Upward growth is restricted by sub-aerial exposure (Burne and Moore, 1993). The oldest parts of the thrombolite reef have been dated at 1950 years BP (Moore and Burne 1994), and Lake Clifton is underlain by sediments of marine origin, the youngest of which are dated at 4,670-3,890 years BP (Coshell and Rosen, 1994).

Samples were collected from a narrow reef along the north-eastern shoreline of the lake (Figure 1c and 1d). Two large, fully submerged whole thrombolites (T2 and T3) were collected from within ~2 m of each other in summer 2008 (Figure 1e and 1f); each thrombolite was split into two halves. Each half was separated into (1) outer surface (outermost 5 mm) and (2) inner surface (the next 5 mm under the outer layer) with 3 replicated subsamples being collected from each thrombolite head to encompass diversity across the head. Samples collected from one half of each thrombolite were immediately frozen in liquid nitrogen for transport back to the laboratory. Samples were stored at -40°C prior to nucleic acid extraction. Samples collected from the second half of each thrombolite were stored at 4°C for microscopy and mineralogical analyses.

Optical Microscopy

Thin sections (30 and 100 um in thickness) were examined under bright-field and reflected light with Nikon Optiophot-2 (biological) and Optiophot-pol (polarizing) microscopes, imaged with a digital camera and processed using AcQuis and Auto-Montage image-capturing software.

Water Chemistry

Water samples were collected from around the thrombolites at the same time as thrombolite sampling. These samples were stored on ice for transport back to the laboratory prior to analysis. All water analyses (Table 1) were performed by the Chemistry Centre of Western Australia (Bentley, Western Australia).

X-ray Diffraction and Inductively Coupled Mass-spectrometry (ICP)

All samples were ground manually using an agate mortar and pestle. Mineral composition of the thrombolite material was identified using powder x-ray diffraction (XRD) using a Phillips PW 1830 x-ray diffractometer with CuKα radiation and diffracted beam monochromator. Patterns were determined for the range 3 - 70° 2θ with a step size of 0.02°. Patterns were smoothed and area under the peak used for semi-quantitative determination of the amount of each mineral present.

Elemental composition of samples was determined using inductively coupled mass-spectrometry (ICP-MS) of diluted HNO_3-digested glass fusion beads generated using 0.1 g of thrombolytic material. Elemental composition (Table 2) was determined using a Perkin Elmer Elan 6000 (ICP-MS) with a flow injection analysis system (Fias 400) and an AS 91 auto analyzer.

DNA Isolation

Each large thrombolite was sectioned into 3 replicated portions in the field and DNA was extracted from duplicated outer 5 mm (T2 and T3 outer) and inner 5 mm (T2 and T3 inner) material of each thrombolite following the method of Griffiths et al. (2003) with slight modifications as follows: samples were lyzed for 2 min, nucleic acids were pelleted by centrifuging at 16 000 × *g* for 15 min and DNA was re-suspended in sterile H_2O. A Qubit ® 2.0 Fluorometer (Life technologies, USA) was used to obtain an accurate quantitation of

DNA. DNA was extracted within 2 months of sample collection and stored at -80°C prior to sequencing.

Ion Torrent PGM Barcoded Sequencing

For each thrombolite sample (T2 and T3; outer and inner surfaces) a 300 b.p. section of the V4 region of the bacterial 16S rRNA gene was amplified by PCR primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Mori et al. 2014). Modification of the forward primer included the addition of a PGM (Personal Genome Machine) sequencing adaptor, a 'GT' spacer and unique error correcting Golay barcode (Hamady et al. 2008) to allow for multiplexed sequencing. Duplicate amplifications were performed for each sample and pooled prior to further analysis. A universal primer mix was first prepared by combining untagged primers with the reverse primer. The untagged reverse primer, 806R, was combined with the untagged forward primer, 515F, to a final concentration of 0.44 µM. For barcoded PCR amplification, template DNA (1 ng) was amplified in a 20 µl volume containing 0.2 µM each of the universal primer mix and the forward tagged primer, 600ng BSA (Life Technologies) and 2.5x 5Prime Hot Master Mix (5 Prime, Australia). All samples were amplified in duplicate and the reaction was run on an Eppendorf Master cycler EP S module (Thermo Fisher Scientific, Victoria) using the following cycling conditions: an initial denaturation at 94 ˚C for 2 min, followed by 25 cycles of denaturation at 94 ˚C for 45 sec, annealing at 50 ˚C for 1 min, elongation at 65 ˚C for 1 min 30 sec, then 7 cycles of denaturation at 94 ˚C for 45 sec and annealing at 65 ˚C for 1 min 30 sec, lastly a final extension at 65 ˚C for 10 min.

Following amplification, all PCR products were checked for size and specificity by gel electrophoresis on a 1.8% w/v agarose gel; PCR products were gel purified, quantified (Qubit - Life Technologies, Australia) and equally pooled for multiplex sequencing on an Ion Torrent Personal Genome Machine. The pooled sample was further purified using Ampure

(Beckman Coulter, Australia) following the manufacturer's protocol and eluted in low TE buffer. After sequencing, the individual sequence reads were filtered within the PGM software to remove low quality and polyclonal sequences; sequences matching the PGM 3' adaptor were also automatically trimmed. All PGM quality filtered data were exported as FastQ files which were split into constituent *.fasta and *.qual files and subsequently analyzed using the QIIME pipeline (version 1.7; http://www.qiime.org; Caporaso et al. 2010). Briefly, the data were subjected to quality control whereby each sequence was screened for an average Q (quality score; $Q \geq 20$), ambiguous bases (count = 0), allowable primer mis-matches (count = 0), homopolymers (length $\leq$ 12) and removal of singleton OTUs (operational taxonomic units). Within the QIIME environment OTU picking was performed using uclust against a subset of the Greenegenes taxonomy database (http://www.greengenes.lbl.gov; version 13_5; DeSantis et al. 2006) filtered at 97% sequence identity. Taxonomy was assigned to each read by accepting the Greenegenes taxonomy string of the best matching Greenegenes sequence. Phylogenetic tree building was performed with FastTree and OTU tables prepared. All potentially chimeric sequences were identified using Usearch61 (Edgar 2010) and removed and a cut-off filter of 0.005% was applied. The sequence data were sub-sampled to 80,000 sequences per sample to ensure comparable estimators across experimental units. Alpha diversity metrics including Shannon diversity (a measure of α-diversity that incorporates relative abundance), Chao1 (a non-parametric richness estimator) and Faith's phylogenetic diversity were calculated after rarefaction. Species diversity is a measure of the diversity within an ecological community that incorporates both species richness (the number of species in a community) and the evenness of species' abundances. Raw demultiplexed reads were deposited in the MG-RAST database (www.metagenomics.anl.gov) under accession number 15755.

Statistical Analysis

Univariate statistical analyses were performed using GenStat (16th edition; Lawes Trust, Harpenden, UK). Analysis of variance (ANOVA) was performed to determine whether thrombolite number (T2 versus T3) or position within the thrombolite (outer surface versus inner surface) significantly affected major element chemistry and microbial measures (relative abundance of major phyla and alpha diversity metrics). To determine whether microbial community structure was significantly impacted by thrombolite sampled (T2 versus T3), or depth (outer surface versus inner surface) multivariate statistical analyses were performed in Primer 6 (Primer-E Ltd., United Kingdom). Analyses were performed on data that had been generated using the QIIME pipeline as described above, with no transformation and applying the Bray-Curtis measure of similarity. Tests of the null hypothesis that there are no differences among *a priori*-defined groups were performed by permutational multivariate analysis of variance (PERMANOVA). To visualise differences ordinations were performed by principal coordinate (PCO) analysis and Pearson correlations of individual elemental variables with PCO axes were also performed.

Results

Microscopy, Mineralogy and Elemental Composition

Thin sections through the outer portions (c. 5 cm) of the two microbialites clearly demonstrate the meso-scale clotted texture characteristic of thrombolites (Figure 2a). Some of these clots contain such high quantities of dark, fluffy organic material that individual organisms are difficult to visualise. However, many clots are rather lighter in colour revealing a microbial community comprising coccoid and filamentous organisms, plus extracellular organic material (sheaths, EPS), set in a porous mineral matrix (Figure 2b). Visually, the most conspicuous members of the community are at least three varieties of large (c. 10-40

μm diameter) filamentous organisms morphologically comparable to cyanobacteria such as *Oscillatoria* sp., *Johannesbaptista* sp. and *Anabaena* sp. (Figure 2c; see also Wacey et al., 2010), plus large (c. 20 μm diameter) coccoids likely also of cyanobacterial affinity. These observations are consistent with previous microscopy-based analyses of Lake Clifton thrombolite communities (e.g., Neil, 1984; Moore et al., 1984; Moore and Burne, 1994; Smith et al., 2010), where cyanobacteria were identified as a dominant component of the microbial community. It is noteworthy that we did not observe well preserved examples of *Scytonema* sp., suggesting a continued drop in relative abundance of this genus that once comprised almost 20% of organisms in the thrombolites (Neil, 1984; Smith et al., 2010) but were recently reported to have declined to <1% (Smith et al., 2010).

XRD analysis showed that thrombolite samples contain only two crystalline phases; aragonite ($CaCO_3$, a calcium carbonate mineral) and halite (NaCl, salt resulting from the surrounding lake water). An unidentified amorphous phase was also noted in these samples; likely the Mg-Si-O phase discussed by Wacey et al. (2010) and identified using NanoSIMS which would also account for the significant presence of magnesium in the chemistry data. Elemental analysis (Table 2) confirmed the presence of calcium, magnesium, and sodium as well as strontium (likely incorporated into the calcium carbonate) and sulfur (potentially from an organic source); this concurs with the detection of calcium carbonate and halite minerals by XRD.

Water Chemistry

Salinity has been continuously increasing at Lake Clifton over the past 20 years. At the time of sampling salinity was measured at 36.7 gl^{-1} (Table 1) which is an increase with time from 18.2 gl^{-1} in 1993; 24.1 gl^{-1} in 2001 and 32.2 gl^{-1} in 2006, all measured at a similar time of year (Smith et al. 2010).

Microbial Community Composition

16S rRNA gene amplicon libraries were generated from each of the samples collected with classification and community analysis based on the defined V4 region of the 16S rRNA gene in all compared reads. The Ion Torrent platform was used to sequence amplicons; this platform produces archaeal and bacterial community profiles highly comparable to 454 sequencing (Yergeau et al., 2012) and has previously been used to characterize microbial populations (Yergeau et al., 2012; Bell et al., 2013a; 2013b). Libraries were clustered into OTUs at 97% similarity or greater. A total of 3.66 million barcoded reads (average length 250bp) were generated, of which 2.07 million unique sequences were recovered after quality filtering. Sequences were sorted by barcode, aligned and analyzed using the QIIME pipeline as described above. Rarified sequence data (80,000 sequence reads per sample) were used to generate diversity and richness indices for both outer and inner surface thrombolite samples from T2 and T3. The number of observed species in the thrombolites was significantly ($p<0.03$) higher in inner surface samples at 3051 compared to 2041 in outer surface samples. Richness values (Chao1) were also high generally with inner surface samples having a significantly ($p<0.02$) higher Chao1 at 3465 compared to 2214 for the outer surface samples. Diversity (Shannon) did not vary significantly between surfaces and was generally high (Shannon diversity, H' = 8.0) and Faith's phylogenetic diversity was significantly ($p<0.03$) higher in the inner surface (264) than the outer surface (192).

Of the total number of sequences analyzed 98.6% were assigned to Bacteria and 1.4% to Archaea. Within the Archaea the dominant phyla represented were the *Euryarchaeota* and the *Crenarchaeota*. Bacterial community structure at this site is displayed in Figure 3, where the distance between points is proportional to the similarity of the bacterial community profiles of those samples (as assessed by Bray-Curtis). Here the PCO plot explains approximately 80% of the total variation (Figure 3) with community structure being significantly ($p<0.05$)

affected by depth (outer surface - top 5mm; and inner surface - 5mm directly underneath) but not by originating sample (T2 and T3). Pearson correlations with the PCO axes (Figure 3) demonstrated that the community structure was positively correlated with calcium, strontium and potassium and negatively correlated with iron and aluminium with PCO axis 1 explaining 66.5% and PCO axis 2 explaining 13.2% of the total variation in community structure. The thrombolitic bacterial sequence libraries were composed of sequences from across 53 different bacterial phyla with the *Proteobacteria* being the most abundant phylum comprising of 51% in outer surface samples and 29% in inner surface samples (Figure 4). Across depth there were significant ($p<0.03$) differences in the relative abundance across a variety of phyla thereafter with the *Actinobacteria*, *Alphaproteobacteria* (subclass of the *Proteobacteria*) and *Cyanobacteria* all higher in relative abundance in outer surface samples and *Firmicutes*, *Spirochaetes* and *Chloroflexi* all higher in relative abundance in the inner surface samples (Figure 4). Phyla with low relative abundance included *Nitrospira*, *Chlamydiae* and *Chlorobi* as well as candidate phyla (organisms for which there are no cultured representative e.g. TM6, OP11 and GN02).

Within the *Proteobacteria* the *Alphaproteobacteria* were significantly ($p<0.05$) more abundant in the outer surface (36%) when compared to the inner surface (15%), both the *Delta-* and *Gammaproteobacteria* were present in equal abundance in the outer surface (8 and 6.5% respectively) and inner surface (7 and 5% respectively). *Beta* and *Epilsonproteobacteria* were present in abundance of less than 1%. Within the *Alphaproteobacteria* the most abundant Orders included *Rhizobiales*, *Rhodospilles*, *Rhodobacterales*, *Sphingomondales* and BD7-3. Only the *Rhizobiales, Rhodospilles* and *Rhodobacterales* were significantly different in relative abundance between outer and inner surfaces. Within the *Deltaproteobacteria* the most abundant Orders included *Myxococcales*, *Desulfobacterales*, *Syntrophobacterales*, NB1-j and PBC076. There were significant

differences in relative abundance between outer surface and inner surface in each of these orders with the exception of *Syntrophobacterales* (Figure 5a). We also identified the presence of the Family *Thermodesulfovibrionaceae* of the *Nitrospirae* in both outer and inner surface samples, although they were present at low relative abundance (< 1%). *Cyanobacteria* were significantly ($p<0.003$) more abundant in outer surface samples at 3.4% relative abundance compared to the inner surface at 0.6% relative abundance, with the coverage including the classes *Oscillatoriophycideae* and *Synechococcophycideae* which were significantly ($p<0.02$) more abundant in outer surface sections and *Gloeobacterophycideae* which were only present in outer surface sections.

Discussion

The thrombolite reef at Lake Clifton is known to support the largest living, non-marine microbialite community in the Southern hemisphere and was placed on the critically endangered list in 2000 (Ramsar Convention). Cyanobacteria were thought to be the dominant phylum present when these thrombolites were last extensively studied in the 1980's and 1990's (Neil, 1984; Moore et al., 1984; Moore 1991; 1993; Burne and Moore 1993). However it should be noted that most historical work was completed using microscopy-based tools and thus the relative abundance of cyanobacteria may have been over estimated. In recent times the lake water quality has been impacted by local development and agriculture and this is expected to have had a dramatic impact on the composition of the microbial populations that are currently present in the reef. Alterations to water quality include increased salinity and an increase in nutrient levels, in particular nitrogen and phosphorus.

In the current study we report a significant change in water chemistry – the salinity on the day we collected our samples (Dec 2008) was 36.7 gL^{-1}. This compares to reported values of 18.2 gL^{-1} in the period 1985-1993; 24.1 gL^{-1} in 1994 and 32.2 gL^{-1} in 2005 (as described in a

study by Smith et al. 2010). This, along with other more comprehensive analyses of water quality at Lake Clifton as measured by the UWA Centre for Water Research (per. comm.) confirms that the salinity increase in Lake Clifton has been significant and is at least equivalent to the salinity of the ocean and can no longer be considered hyposaline; this likely has had effect on the inhabitant thrombolite microbial populations. The second noteworthy point is that nitrogen levels have also increased in this time which has led to the lake water having a higher nutrient load that was reported in earlier studies. Although in early microscopy studies Moore and colleagues reported (1984) that the thrombolites at Lake Clifton were dominated by *Scytonema* (Order *Nostoccales*) which are known to require both low salinity and low nutrient levels, a similar microscopy study by Smith and colleagues in 2010 reported that *Scytonema* were no longer the dominant organism at Lake Clifton.

Here we report that the thrombolites of Lake Clifton are dominated by bacteria (98.6%) with archaea making up only 1.4% of the community. This is in agreement with studies of thrombolites at other locations (e.g. Mexico and Bahamas) where bacteria also dominate and archaea were reported at 0.4-1.7% relative abundance (Centeno et al. 2012; Mobberley et al. 2012). The most dominant phylum represented in Lake Clifton thrombolites were the *Proteobacteria*, both in the outer and inner surface sections of sampled thrombolytic material. This is in agreement with other studies where *Proteobacteria* have been observed to be the dominant phylum present in non-marine microbialites across a variety of locations in Mexico (Centeno et al. 2012) plus marine thrombolite microbial mat communities (black, beige, pink and button mats) from Highborne Cay in The Bahamas (Mobberley et al. 2012). In both these studies the A*lphaproteobacteria* were the most dominant – this is also in agreement with the current study. It is likely that these Alphaproteobacterial phylotypes, as well as containing potential photosynthesizing members, display diverse metabolisms from autotrophy to

heterotrophy, which together with their richness, suggests an important role in thrombolite organization and activity.

Chloroflexi were present in relatively high numbers (5 and 7% in outer and inner surface sections respectively) and may also contribute to the photosynthesizing capacity of the community. In addition to the dominant *Alphaproteobacteria* and *Chloroflexi* photosynthesizers, we report the presence of typical heterotrophs including *Bacteroidetes* in both outer surface (19%) and inner surface (13%) sections and small communities of *Planctomycetes* (3.5% on average), and we also report significant populations of *Firmicutes* in the inner surface sections (24%). Previous studies have reported the bacterial diversity of giant microbialites from the sodic but low salinity Lake Van (Eastern Anatolia, Turkey; Lopez-Garcia et al. 2005), where the most abundant and diverse lineages were *Firmicutes* followed by *Proteobacteria*, *Cyanobacteria* and *Actinobacteria*. In agreement with other studies (Centeno et al. 2012; Mobberley et al. 2012) we report the presence of phyla of low abundance including *Actinobacteria, Nitrospira, Chlamydiae, Spirochaetes, Chloriobi, Fusobacteria, Gemmatimonadetes* as well as candidate phyla (e.g. TM7, OP10, SR1) which were represented by a few sequences only.

Cyanobacteria were present at low abundance in the current study in both outer surface (3%) and inner surface (1%) sections. This again is in agreement with a variety of other studies where there is site specific reporting of low abundance of *Cyanobacteria*, for example Centeno (2012) where only one location had high relative abundance of *Cyanobacteria* (24%) and all others were significantly lower (of the order 1-8%) and similar in relative abundance to that reported here. Within the Cyanobacteria we report the presence of *Synechoccophycidea* (Orders *Pseudanabaenales* and *Synechococcales*) and *Oscillatoriophycideae* (Orders *Chroococcales* and Oscillatorales) as the dominant classes;

however we emphasise that the total relative abundance of *Cyanobacteria* at this site at the time of sampling was low overall (1-3%).

Thrombolite formation likely is dictated by the metabolisms of the organisms present; this will be the net result of microbial metabolic activities which favour either carbonate dissolution or precipitation (Dupraz et al. 2009). Bacterial photosynthesis likely leads to consumption of bicarbonate resulting in carbonate precipitation (Dupraz et al. 2009). Anoxygenic photosynthetic bacteria, some of which may also be sulphide oxidizers (for example organisms identified here within the *Chloroflexi* and the Orders *Rhodobacterales* and *Rhizobiales* of the *Alphaproteobacteria*) may also induce carbonate precipitation (Bostak et al. 2007, Couradeau et al. 2011). Anoxygenic photosynthesis consumes hydrogen sulphide (H_2S), which is likely produced by the activity of sulfate reducing bacteria (for example those identified here within the Family *Thermodesulfovibrionaceae* of the *Nitrospirae* and the Order *Desulfobacterales* of the *Gammaproteobacteria*). Sulfate reduction generates carbonate ions potentially leading to carbonate precipitation (Baumgartner et al. 2006). Heterotrophic bacteria, including those identified here within the *Planctomycetes Bacteroidetes* and *Acidobacteria* may induce carbonate dissolution due to respiration of organic matter and production of protons (Dupraz and Visscher 2005). Sulfur oxidising bacteria, for example those identified here within the *Chromatiales* of the *Gammaproteobacteria,* may also promote carbonate dissolution. At our study site it is likely that in general both photosynthesis and sulfate reduction processes are promoting carbonate precipitation with aerobic respiration and sulphide oxidation promoting carbonate dissolution. The net carbonate precipitation depends on the balance between these microbial metabolic activities.

Extracellular polymeric substance (EPS) production by the microbial community is also critical for thrombolite formation (Mobberley et al. 2015) as it can serve as a carbon source

for heterotrophic metabolism (Decho et al. 2005) and functions as a nucleation site for calcium carbonate precipitation (Kawaguchi et al. 2000). Although cyanobacteria are generally recognized as the most important EPS producers (Dupraz et al. 2009) the role of heterotrophic bacteria such as sulfate reducing bacteria (including those identified here within the Family *Thermodesulfovibrionaceae* of the *Nitrospirae* and the Order *Desulfobacterales* of the *Gammaproteobacteria*) in the production of EPS has been demonstrated (Braissant et al. 2007).

This is the first study to utilize molecular approaches to characterize the microbial populations that form the Lake Clifton thrombolites. We have demonstrated that the system is dominated by proteobacteria, in particular the *Alphaproteobacteria* with cyanobacteria only present at a relatively low abundance. As previous work characterising the organisms at this site utilised microscopy-based tools it is likely that the numbers of cyanobacteria were overestimated (e.g., Neil, 1984; Moore et al., 1984; Moore 1993). Nonetheless, given the lake conditions at the time, it is reasonable to assume that cyanobacteria were more numerous some 20-30 years ago than currently recorded. The current study reinforces the suggestion from the microscopy-based study of Smith et al. (2010) that cyanobacteria are no longer the dominant organisms at the lake. We can conclude that the changing physico-chemical conditions at Lake Clifton may have contributed to a decline in the cyanobacterial populations thought to be fundamental to thrombolite formation in this system; however further work is required to elucidate the underlying mechanisms that will be impacted by such changes.

Figure and Table legends

Figure. 1. Location of sampled thrombolites from Lake Clifton (a) Map showing location of Lake Clifton, approximately 100 km south of Perth in Western Australia. A narrow reef of thrombolites is found along the north-eastern shoreline of the lake, extending for around 6 km (b) Variation in thrombolite morphology with water depth. Star indicates approximate sampling site (c) Views looking north (d) and west of the thrombolite reef. Stars indicate sampling locality, where two thrombolite heads were collected from within ~2 m of one another (e) and (f) Part section of living thrombolites collected form permanently submerged edge of the thrombolite reef (samples T2 and T3).

Figure. 2. Microtextures of the living thrombolites (a) Scan of a petrographic thin section through the outer ~5 cm of sample T2. Irregular lithified mesoclots (green/brown) are interspersed with cavities (white) containing detrital sediment and now infilled with resin (b) Transmitted light microscopy image of the visible microbial community within one of the mesoclots. Fluffy carbonaceous material is common, together with coccoid and filamentous bacteria. Large filamentous cyanobacteria stand out in optical microscopy and this may have led to an overestimation of their importance in previous studies. (c) Transmitted light microscopy image showing well preserved cyanobacterial trichomes and sheaths in a matrix of aragonite.

Figure. 3. Principal coordinate (PCO) analysis of bacterial community structure by barcoded sequencing where closed triangles represent the outer surface and open triangles represent the inner surface thrombolytic material. T2 and T3 refer to sampled thrombolite heads. Vectors show Pearson correlations with elemental characteristics of the thrombolitic material.

Figure. 4. Comparison of thrombolite bacterial and archaeal community composition showing dominant phyla based on the Greengenes taxonomy (version 13_5). Significant differences are indicated (*$p<0.05$; **$p<0.005$); n=3.

Figure 5. Comparison of thrombolite Proteobacterial Classes based on the Greengenes taxonomy (version 13_5). Significant differences are indicated (*p<0.05; **p<0.005); n=3.

Table 1. Water chemistry data from the thrombolite sampling site on the eastern margin of the lake.

Table 2. Elemental composition of the thrombolite material as characterised by inductively coupled mass-spectrometry. Significant differences are indicated (**p<0.005); n=3.

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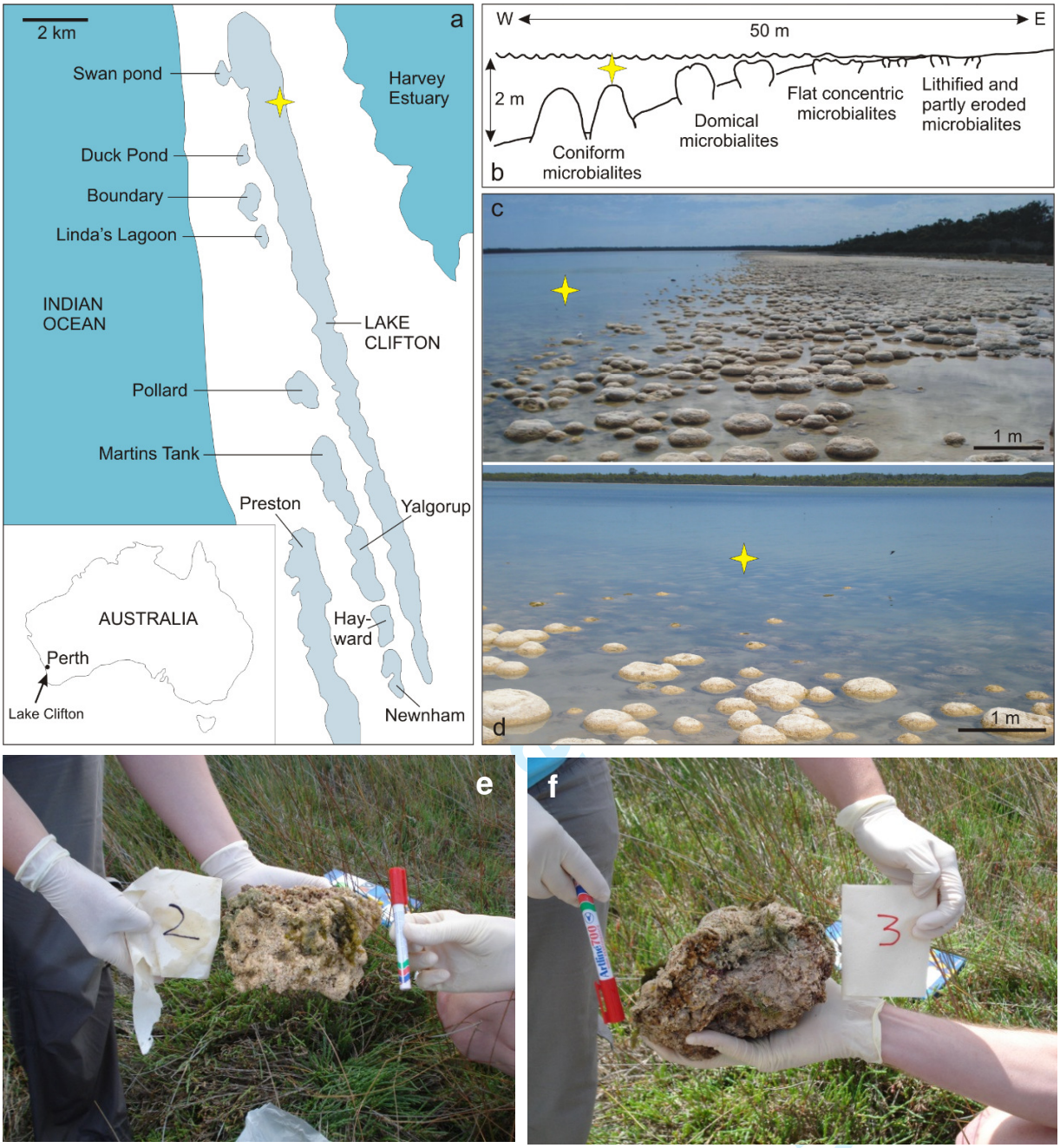
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Duck Pond
Boundary
Linda's Lagoon
INDIAN OCEAN
Harvey Estuary
LAKE CLIFTON
Pollard
Martins Tank
Preston
Yalgorup
Hay-ward
Newnham
AUSTRALIA
Perth
Lake Clifton
b
W
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50 m
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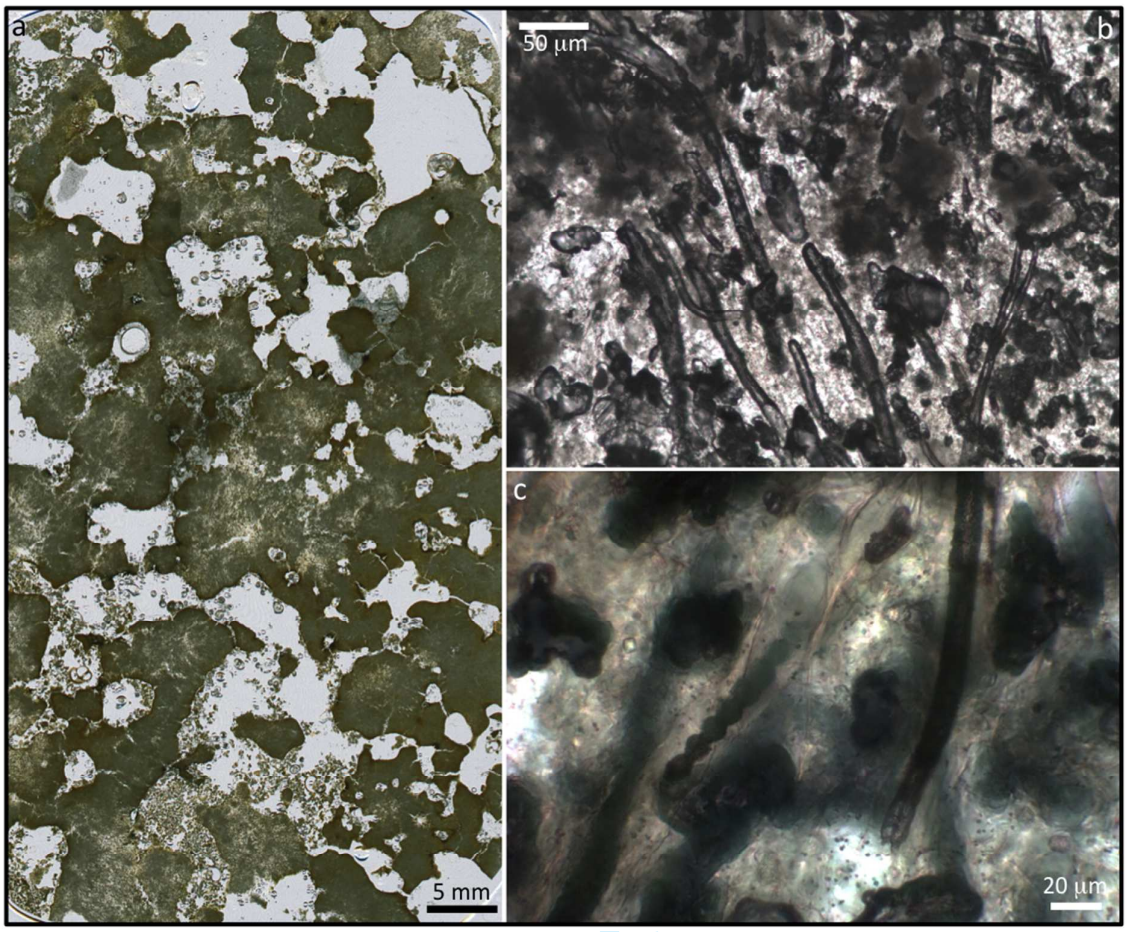

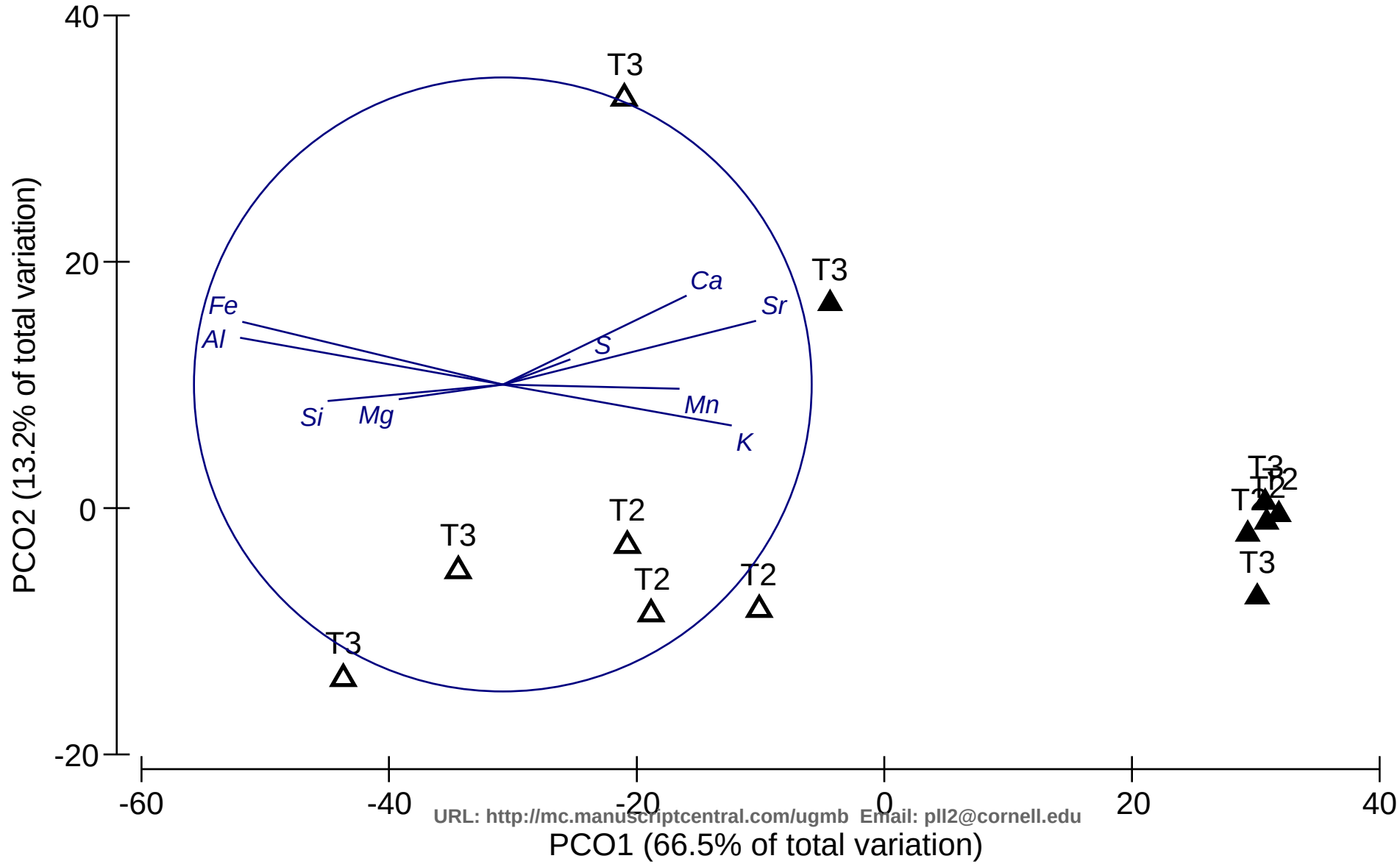
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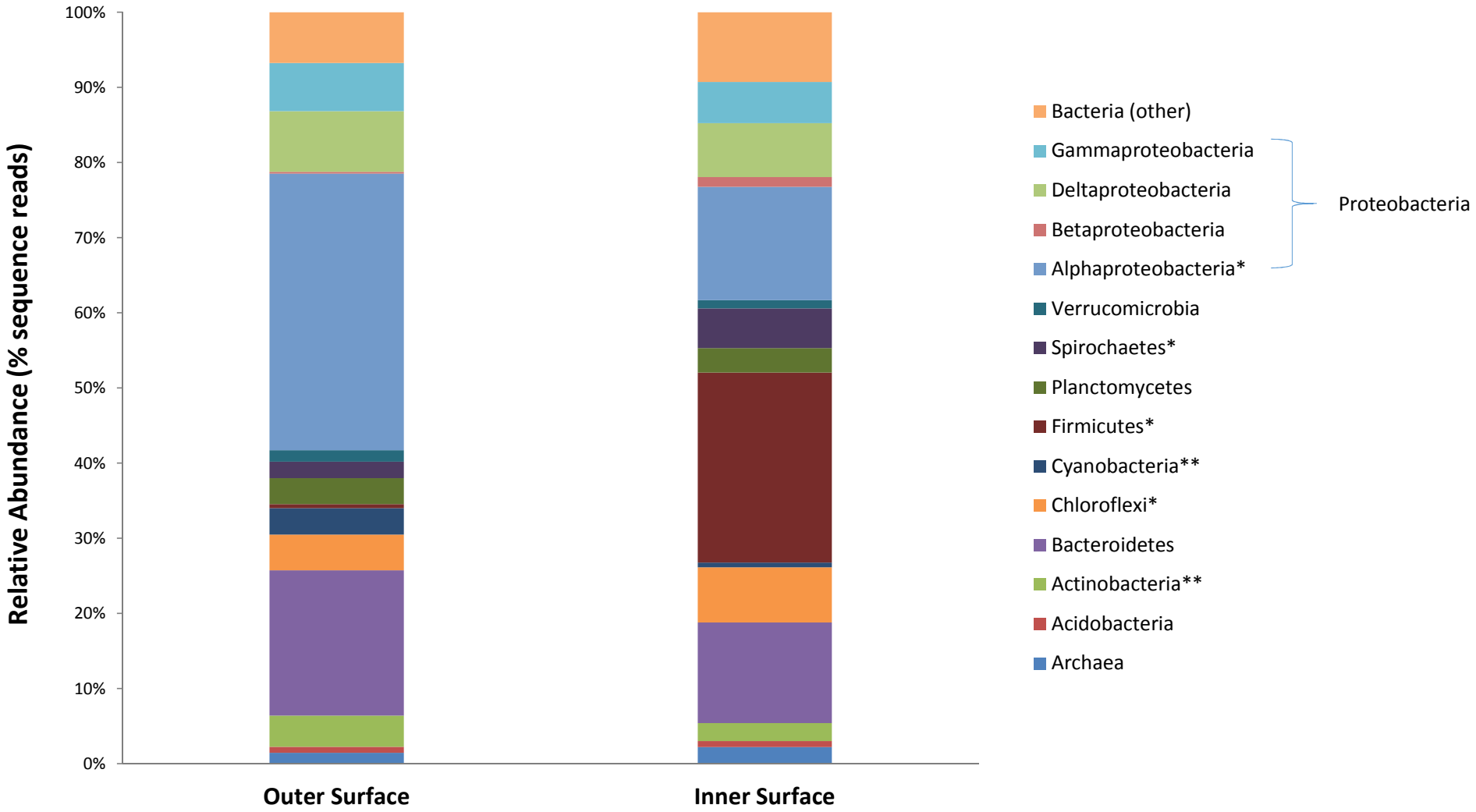
Relative Abundance (% sequence reads)
100%
90%
80%
70%
60%
50%
40%
30%
20%
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Outer Surface
Inner Surface
Bacteria (other)
Gammaproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria*
Proteobacteria
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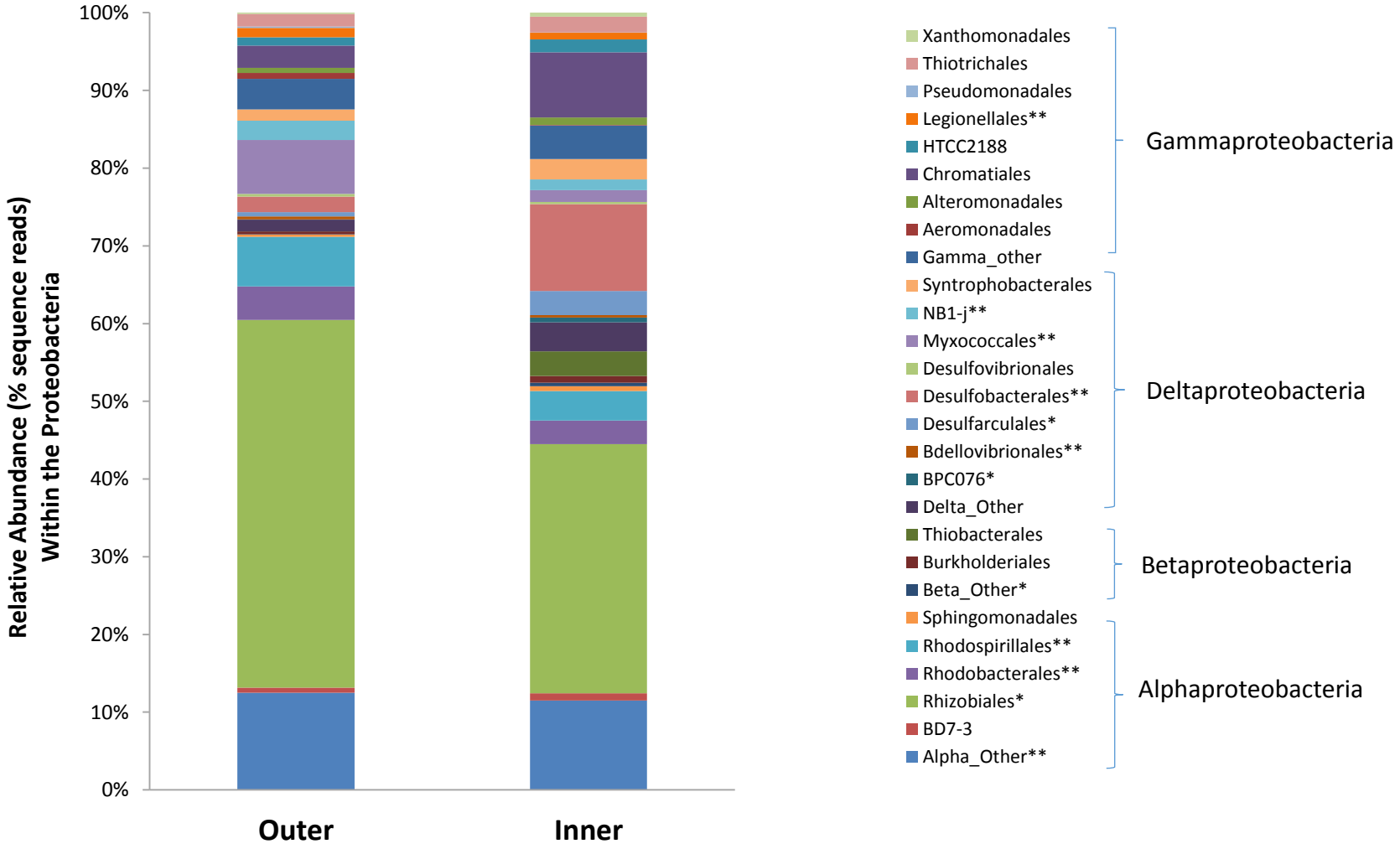
Relative Abundance (% sequence reads) Within the Proteobacteria
100%
90%
80%
70%
60%
50%
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30%
20%
10%
0%
Outer
Inner
Xanthomonadales
Thiotrichales
Pseudomonadales
Legionellales**
HTCC2188
Chromatiales
Alteromonadales
Aeromonadales
Gamma_other
Gammaproteobacteria
Syntrophobacterales
NB1-j**
Myxococcales**
Desulfovibrionales
Desulfobacterales**
Desulfarculales*
Bdellovibrionales**
BPC076*
Delta_Other
Deltaproteobacteria
Thiobacterales
Burkholderiales
Beta_Other*
Betaproteobacteria
Sphingomonadales
Rhodospirillales**
Rhodobacterales**
Rhizobiales*
BD7-3
Alpha_Other**
Alphaproteobacteria

Water Chemistry (mg L^{-1} unless otherwise stated)	Mean
pH	8.3
EC (mS m^{-1})	6653
Total Dissolved Salts (g L^{-1})	36.7
Turbidity (NTU)	16
Alkalinity (as $CaCO_3$)	114
Carbonate	12
Bicarbonate	123
Calcium	723
Magnesium	1760
Nitrogen	2.8
Sulphur	977
Total Inorganic Carbon	19
Total Organic Carbon	36

Elemental Analysis (mg kg^{-1})	Outer Surface	Inner Surface
Al**	29	163
Ba**	101	104
Ca**	146801	164180
Fe**	46	93
K**	728	470
Mg**	18716	17917
Na	12842	12033
S*	5117	2697
Sr	1476	1444

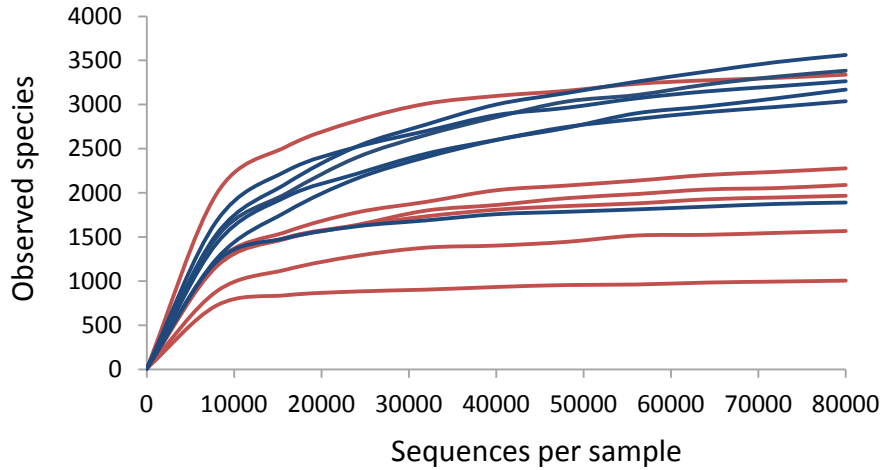

Figure S1 Rarefaction curve detailing number of observed species per sample sequence output. Outer surface = red; inner surface = blue.